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Isolation, cDNA Cloning, and Characterization of an 18-kDa Hemagglutinin and Amebocyte Aggregation Factor from *Limulus polyphemus* *

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An 18-kDa hemagglutinin which possesses the property of inducing both aggregation of amebocytes and agglutination of erythrocytes has been isolated from *Limulus polyphemus* amebocytes and purified by ion exchange chromatography. This nonglycosylated, single chain polypeptide with an M_r of 18,506 and isoelectric point of 8.3 is stored exclusively in the large secretory granules of amebocytes. Based on the partial N-terminal amino acid sequence of 63 residues, DNA probes have been synthesized for screening a pBR322 cDNA library constructed from *Limulus* amebocytes. The cDNA coding for this protein reveals the presence of a 19-residue signal peptide preceding the 153-residue open reading frame. Northern blot analysis indicates the presence of a single mRNA species. The primary structure derived from the corresponding cDNA sequence reveals an internal homology consisting of two consensus sequences, Val-Asn-Asp/Ser-Trp-Asp and Glu-Asp-Arg-Arg-Trp. The formation of 5 disulfide bonds between 10 half-cysteines divides the molecule into three looped domains each containing the Glu-Asp-Arg-Arg-Trp repeating unit. One of the novel features of this protein is that it shares 37% identity with a 22-kDa mammalian extracellular matrix protein isolated from fetal bovine skin (Neame, P. J., Choi, H. U., and Rosenberg, L. C. (1989) *J. Biol. Chem.* 264, 5474–5479). The two proteins exhibit a similar pattern of looped domains, each domain containing a homologous consensus sequence (*i.e.* Glu-Asp-Arg-Arg-Trp). The overall structure of both proteins seems to be highly related, with the exception of an N-terminal tyrosine-rich region present only in the mammalian extracellular matrix protein. The functional properties of the two proteins are similar in that the *Limulus* 18-kDa protein agglutinates horse erythrocytes and aggregates *Limulus* amebocytes, and the mammalian 22-kDa protein is an effective adhesion promoter for dermal fibroblasts. On the basis of these unique properties, the newly characterized hemagglutinin has been termed *Limulus* 18K agglutination-aggregation factor (18K-LAF).

During the course of our investigation on the components of the *Limulus* host defense system, we have isolated and characterized C-reactive protein (CRP)[1] from the circulating hemolymph of *Limulus polyphemus* (1, 2). Although CRP shares numerous properties with immunoglobulins (3–7), a specific role has yet to be assigned to this molecule. In contrast to CRP and other extracellular hemagglutinins which have been studied extensively, relatively little is known about the existence of intracellular forms of agglutinins in invertebrates. Nevertheless, several intracellular proteins have been isolated from horseshoe crab amebocytes, the majority of which are located in the secretory granules and appear to be related to functional roles of defense and homeostasis. These include proteins that participate in the clotting cascade (8, 9), molecules that exhibit antimicrobial activity (10, 11), and protease inhibitors (12, 13).

A substantial body of literature exists providing considerable evidence that amebocytes are highly sensitive to minimal amounts of endotoxin and that degranulation occurs immediately following exposure to these substances. However, it has been reported that amebocytes also undergo exocytosis in the absence of endotoxin (14). As a result of degranulation, the cells flatten and aggregate, a phenomenon that has also been observed after ionophore-induced exocytosis (15). These findings support the hypothesis that the aggregating factors are intracellular and encounter their receptors on the cell surface after extrusion, promoting homotypic aggregation of the amebocytes. There is also ample evidence suggesting that aggregation does not require serum components, since amebocytes in culture still retain the ability to aggregate (14). The isolation of an intracellular agglutinin and amebocyte aggregation factor has been reported from the amebocytes of the Indian horseshoe crab *Carcinoscorpius rotundicauda* (16). Recently, a 55-kDa protein (*i.e.* limunectin) with adhesion-promoting properties (17) and a 12-kDa endotoxin binding protein (*i.e.* LEBP-PI) with protease inhibitory activity (13) have been cloned and characterized.

This paper describes the purification, cDNA cloning, and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M96983.

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[1] The abbreviations used are: CRP, C-reactive protein; 18K-LAF, *Limulus* 18-kDa agglutination-aggregation factor; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); kbp, kilobase pair(s); bp, base pair(s); HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin; PBS, phosphate-buffered saline; Me_2SO, dimethyl sulfoxide; BSA, bovine serum albumin; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

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characterization of an 18-kDa hemagglutinin and amebocyte aggregation factor (18K-LAF) from the horseshoe crab *L. polyphemus*. Unlike the aforementioned intracellular components, which seem to be unique to *Limulus*, 18K-LAF is related structurally and functionally to a 22-kDa mammalian extracellular matrix protein described by Neame *et al.* (18) and Levandowska *et al.* (19). The 18-kDa *Limulus* protein also exhibits the agglutinating-aggregating properties reported previously for the *Carcinoscorpius* agglutination-aggregation factor isolated by Srimal *et al.* (16).

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Isolation and Purification of 18K-LAF—The purification of 18K-LAF by ion exchange and gel filtration chromatography is summarized in Fig. 1. After unbound proteins migrated through SP-Sephadex C-25, the bound molecules were eluted by a stepwise increase in ionic strength (Fig. 1*A*). Fractions which agglutinated horse erythrocytes (indicated by the presence of a *bar*) were pooled and further fractionated by CM-Sepharose CL-6B ion exchange chromatography (Fig. 1*B*) and Sephadex G-50 (Fig. 1*C*). Fractions exhibiting hemagglutinating activity were analyzed by SDS-PAGE (Fig. 1*D*). The hemagglutinin consists of a single nonglycosylated polypeptide chain with an apparent M_r of 18,000. The yield of protein was approximately 0.1 mg/g of wet amebocytes.

Screening of Limulus cDNA Library—Based on the partial N-terminal amino acid sequence for 18K-LAF (Table I), two degenerate oligodeoxyribonucleotide probes (Fig. 2) corresponding to the mRNA strand (probe II) and antisense strand (probe I) were synthesized. These probes were subsequently employed to screen a pBR322 cDNA library for the presence of the mRNA coding for 18K-LAF. A pBR322 cDNA library was constructed from *Limulus* amebocytes and screened for the presence of the mRNA encoding 18K-LAF. From approximately 30,000 tetracycline-resistant, ampicillin-sensitive recombinant colonies, we identified 18 colonies which hybridized to probe I under stringent hybridization and washing conditions. Each colony was individually selected and rescreened twice, using duplicate filters to minimize false positive signals. After the third screening, five clones were chosen and designated pf12, p37, pf15, p42, and pf22. The clones contained inserts which increased in size from 0.3 kbp (pf12) to 0.85 kbp (pf22). Restriction enzyme mapping of recombinant DNAs with the longest inserts and autoradiography of the corresponding Southern blot (Fig. 3) gave patterns which were indistinguishable for pf15 and p42. Recombinant DNA from clone pf22 (which had the longest insert) was sequenced. Based on the information generated by DNA sequence analysis, additional single sequence probes were synthesized. One of these (*i.e.* probe III, Fig. 2) corresponded to 6 amino acid residues of the leader peptide and was used in the selection of recombinant M13 clones carrying the 5′-untranslated region.

DNA Sequencing—DNA from recombinant plasmids was purified and digested for 2 h with *Pst*I. The entire digest was subcloned into the replicative forms of M13mp18 and M13mp19 at the *Pst*I site. β-Galactosidase-negative plaques which hybridized to probes I or II were selected. Single-stranded recombinant phage DNA was prepared for each plaque, and the deoxyribonucleotide probes I and II were used as primers for dideoxyribonucleotide chain termination sequencing. Three clones (*i.e.* p37, pf15, and pf22) were sequenced to completion and provided overlapping regions. The sequence strategy is illustrated in Fig. 4. In conjunction with probes I and II, additional primers complementary to the plus or minus strand were prepared to sequence the three clones in both directions.

Nucleotide Sequence and Deduced Amino Acid Sequence of 18K-LAF—The nucleotide sequence (Fig. 5) revealed the presence of a 19-residue leader peptide, preceding a mature protein of 153 residues. Stop codon TAA and poly(A) addition signal were located 159 and 16 nucleotides upstream from the beginning of the poly(A) tail, respectively. The overlapping nucleotide sequences between pf15, pf22, and p42 were identical. The amino acid sequence deduced from the corresponding cDNA sequence (Fig. 5) revealed an internal homology and indicated the presence of two internal repeating units, Val-Asn-Asp/Ser-Trp-Asp and Glu-Asp-Arg-Arg-Trp. Each consensus sequence was repeated three times at positions 2, 54, and 110 and 31, 83, and 139, respectively. The formation of five disulfide bonds divides the molecule into three looped domains, each containing the Glu-Asp-Arg-Arg-Trp repeat (Fig. 6). Moreover, the amino acid composition of 18K-LAF deduced from both the N-terminal and cDNA sequence indicates the absence of methionine and the presence of 10 half-cysteines, 32 basic amino acids, and 22 acidic amino acids (Table II). The number of cysteine residues represents 6.5% of the total number of amino acids in the molecule.

Northern Blot Analysis of Limulus RNA—To determine the size of the messenger RNA coding for 18K-LAF, poly(A$^+$) RNA was fractionated on a formaldehyde-denatured agarose gel with DNA fragments and *Escherichia coli* ribosomal RNAs as size markers. RNA was transferred to a nitrocellulose filter

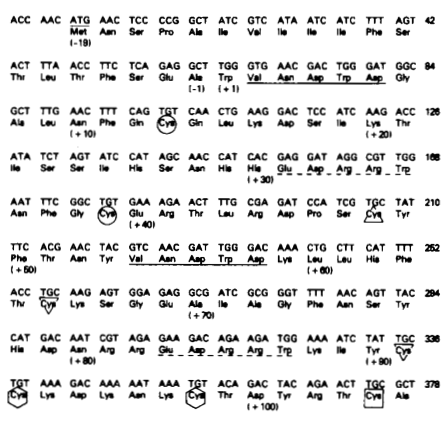


FIG. 5. **Nucleotide and deduced amino acid sequence of 18K-LAF.** The amino acid sequence is listed under the nucleotide sequence. The initiation codon ATG, termination codon TAA, and consensus polyadenylation signal AATAAA are *underlined*. Each pair of cysteine residues linked by disulfide bonds are labeled with identical symbols. Internal homologous sequences are *underlined* in *solid* (Val-Asn-Asp/Ser-Trp-Asp) and *dashed lines* (Glu-Asp-Arg-Arg-Trp). *Numbers in parentheses* represent positions of amino acids in the sequence; residues (−19) to (−1) and (+1) to (+153) correspond to the signal peptide and mature protein, respectively.

[2] Portions of this manuscript (including "Experimental Procedures," Figs. 1–4, 6, 7, 9, and 10, and Tables I-VI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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and hybridized as described under "Experimental Procedures." A single RNA species (*e.g.* <1.0 kb) migrating under 16 S ribosomal RNA was observed (Fig. 7).

Primer Extension of the 5′-End of the Messenger RNA—To determine the length of the 5′-noncoding region, a single-stranded oligodeoxyribonucleotide primer complementary to the DNA sequence coding for residues 1–6 of the mature protein was annealed to poly(A^+) RNA. The primer was elongated in the presence of reverse transcriptase and fractionated on a DNA sequencing gel. The extended nucleotide was approximately 183 bp in length (Fig. 8), and the complete 5′-noncoding region comprised approximately 111 bp. The size of the mRNA from Northern blot analysis was in agreement with a 5′-untranslated region of 111 bp, a leader peptide of 57 bp, a coding sequence of 459 bp, a 3′-untranslated region of 162 bp, and a poly(A) tail of 100–200 bp.

Southern Blot Analysis—Total genomic DNA was digested to completion with the restriction endonuclease *Bgl*II and fractionated on agarose gel. Results of Southern blot analysis indicated the presence of a single gene coding for 18K-LAF (Fig. 9).

Position of Disulfide Bonds—All half-cystine residues of 18K-LAF are linked by disulfide bonds. The positions of disulfide bonds have been established by sequence analysis of peptides generated by thermolysin digestion (Fig. 10 and Tables III-VI). The following disulfide linkages were determined: Cys^{13}-Cys^{39}, Cys^{48}-Cys^{153}, Cys^{65}-Cys^{91}, and Cys^{104}-Cys^{148}. Since incubation of a nonreduced sample with [^{14}C] iodoacetamide did not generate carboxymethylcysteine, we concluded that the remaining half-cystine residues (*i.e.* Cys^{92}-Cys^{98}) were also linked by a disulfide bond.

Immunological Analysis—Reaction of 18K-LAF with rabbit anti-18K-LAF antibody exhibited a single precipitin line and did not cross-react with other components from both *Limulus* amebocyte lysate and hemolymph, which was confirmed by Western blot analysis (not shown).

Cellular Localization—Immunocytochemistry analysis indicated that 18K-LAF is localized exclusively in the large dense secretory granules of the amebocytes and absent from the nucleus and other cytoplasmic organelles (Fig. 11).

Agglutination and Aggregation Studies—18K-LAF promoted the agglutination of heparinized horse erythrocytes at concentrations as low as 10 ng/ml. Other purified proteins from *Limulus* amebocytes and/or coagulogen exhibited no agglutination activity, even at relatively high concentrations (*e.g.* 2 mg/ml). The hemagglutination activity of the pure protein was not inhibited by *Limulus* coagulogen, BSA, or the carbohydrates tested (*e.g.* D-(+)-glucosamine HCl, *N*-acetylglucosamine, *N*-acetylneuraminic acid, glycuronic, and galacturonic acids). At concentrations in the range of 15–116 μg, 18K-LAF also promoted the aggregation of *Limulus* amebocytes which had been collected in the presence of *N*-ethylmaleimide and fixed in formalin (Fig. 12). The aggregation was accompanied by an alteration in cell shape.

Computer Analysis of Sequence Homologies—Comparison of the deduced amino acid sequence for 18K-LAF with those in the GenBank indicated significant homology to a 22-kDa mammalian extracellular matrix protein from fetal bovine skin (18). The analysis reveals a high degree of overall struc-

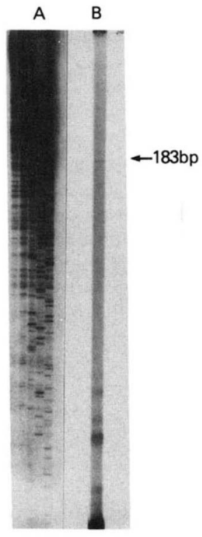


FIG. 8. **Primer extension of the 5′-end of the mRNA.** A single-stranded 17-base oligodeoxyribonucleotide primer complementary to the mRNA and coding for amino acid residues 1–6 of the mature protein as described under "Experimental Procedures." Template: 3 μg of poly(A^+) RNA; annealing conditions: 0.04 M KCl, 10 mM Tris-HCl, pH 8.3, 1 mM EDTA, 40 units of RNasin, 65 °C, 15 min; extension conditions: 10 units of reverse transcriptase, 42 °C, 1 h. *Lane A*, size markers; *lane B*, 2 μl of boiled reaction mixture; *arrow* and *number* correspond to the position and size of the elongated primer.

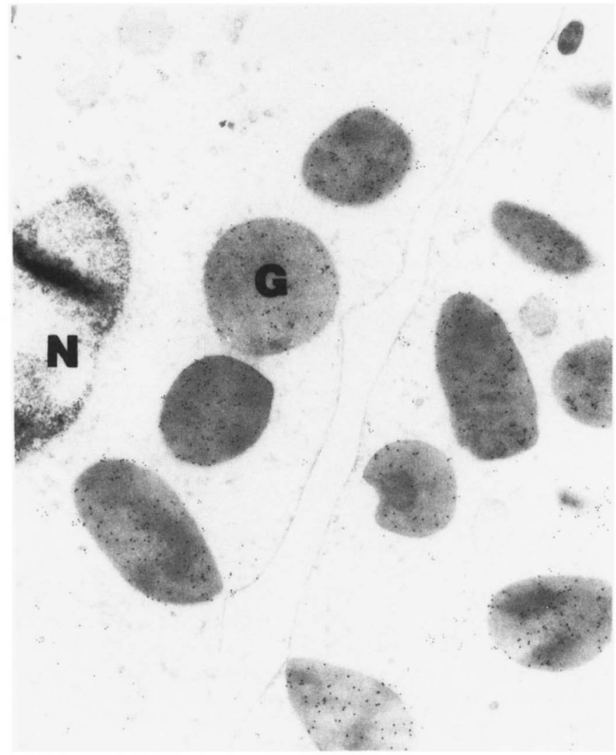


FIG. 11. **Immunocytochemistry studies.** Glutaraldehyde-fixed *Limulus* amebocytes; 1st antibody, affinity-purified rabbit anti-18K-LAF; 2nd antibody, gold-labeled anti-rabbit IgG; Magnification, 8000×; *G*, granule; *N*, nucleus.

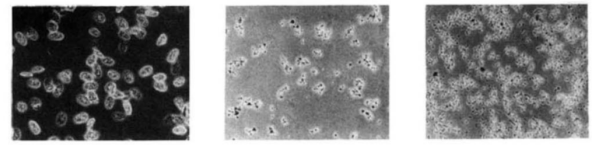


FIG. 12. **Aggregation studies.** Formalin-fixed *Limulus* amebocytes (*A*). Amebocytes in the presence of 15 μg (*B*) and 116 μg (*C*) of 18K-LAF; magnification, Zeiss Axiovert 405M: 400× (*A*) and 80× (*B*, *C*).

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tural identity between the two proteins, including complete conservation of the number and location of all 10 cysteine residues (Fig. 13). The repeating sequence found in the mammalian protein (*i.e.* Asp-Arg-Glx-Trp-Asn/Gln/Lys-Phe/Tyr) shares 95% similarity with one of the consensus sequences of 18K-LAF. A significant difference between both proteins is the presence of an extra 19-amino acid sequence comprising a tyrosine-rich domain in the N-terminal region of the mammalian protein.

DISCUSSION

Although the precise physiological role of lectins remains to be established, their ability to agglutinate cells has long been demonstrated. In this regard, several lectins with hemagglutinin activity have been identified in invertebrate species, including the hemolymph of the horseshoe crab *L. polyphemus* (20–22). In species which do not possess immunoglobulins, these classes of molecules are assumed to play important roles in defense and to assist in the recognition and destruction of invading microorganisms. As part of our ongoing investigation on invertebrate defense systems, we have isolated and characterized an 18-kDa protein with both hemagglutination and aggregation activities. The 18K-LAF hemagglutinin has been purified from *Limulus* amebocyte lysate by ion exchange chromatography on SP-Sephadex C-25 followed by CM-Sepharose CL-6B. The amino acid sequence deduced from the corresponding cDNA sequence (Fig. 5) is in agreement with the partial N-terminal sequence generated by automated Edman degradation of the intact protein (Table I). The cDNA sequence reveals the presence of a 19-residue peptide which exhibits the characteristics of a signal peptide (23), particularly a stretch of hydrophobic residues between the initiator methionine and the site of proteolytic cleavage leading to the mature protein.

The 153-residue mature protein contains 32 basic amino acids, 22 acidic amino acids, and 10 half-cysteines forming five disulfide bonds (Fig. 5). The following disulfide bonds have been determined by sequence analysis of disulfide-linked peptides generated by digestion of the protein with thermolysin (Fig. 10): Cys^{13}-Cys^{39}, Cys^{48}-Cys^{153}, Cys^{65}-Cys^{91}, and Cys^{104}-Cys^{148} (Tables III-VI). The fifth disulfide bond between Cys^{92}-Cys^{98} was assigned by default since there is no free cysteine in the molecule. The absence of free sulfhydryl groups in 18K-LAF is supported by the fact that no carboxymethylcysteine derivative is generated upon incubation of the intact molecule with radiolabeled [^{14}C]iodoacetamide. The most intriguing feature of 18K-LAF is the existence of an internal

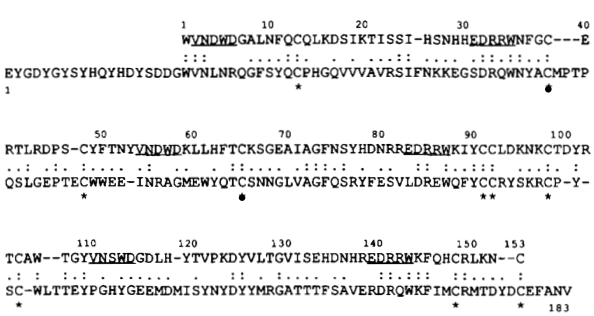


FIG. 13. **Sequence comparison between 18K-LAF *versus* 22-kDa extracellular matrix protein from fetal bovine skin.** Optimal alignment of the deduced amino acid sequence of 18K-LAF with the mammalian 22-kDa extracellular matrix protein, based on the FASTA program (24). Identical amino acids are connected by a *colon* and conservative replacements by a *full stop*. Gaps imposed to maximize alignment are indicated by *dashes*. The cysteines, which are conserved in both proteins, are highlighted by *asterisks*.

homology which is mainly demonstrated by the presence of two consensus sequences, Val-Asn-Asp/Ser-Trp-Asp and Glu-Asp-Arg-Arg-Trp at positions 2, 54, 110 and 31, 83, 139, respectively. The disulfide bonds between Cys^{13}-Cys^{39}, Cys^{65}-Cys^{91}, and Cys^{104}-Cys^{148} divide the molecule into three looped domains (Fig. 6), each containing the Glu-Asp-Arg-Arg-Trp repeat.

Comparison of the amino acid sequence of 18K-LAF with all known protein sequences by the FASTA program (24) resulted in several relevant observations: 1) the protein shares 37% overall identity with a 22-kDa mammalian extracellular matrix protein (18) from fetal bovine skin (Fig. 13); 2) the short repeat Glu-Asp-Arg-Arg-Trp is highly conserved in both proteins; 3) the numbers and positions of the 10 half-cysteine residues are conserved; and 4) the N-terminal domain comprising a 19-amino acid tyrosine-rich region in the mammalian protein is absent in 18K-LAF. Despite these basic structural similarities, there is a major difference in the physicochemical properties of the two proteins. Specifically, the 18K-LAF hemagglutinin is a basic protein with a pI of 8.3, whereas the 22-kDa extracellular matrix protein is acidic (*i.e.* pI = 4.6).

Data on the sequence analysis of *Limulus* proteins reveal that although significant similarities have been detected in several classes of proteins such as CRP (1, 2), clotting factors (8, 9), and protease inhibitors (*e.g.* mainly α_2-macroglobulin) (25) with their vertebrate counterparts, these similarities are often restricted to short stretches and limited regions. Although *Limulus* 18K-LAF and the 22-kDa mammalian extracellular matrix protein share only 37% overall sequence identity, several key structural and functional aspects including their participation in cell adhesion and/or aggregation appear to be highly conserved. The observed conservation in structure and biological activity between these proteins, one originating from invertebrate and the other from vertebrate species, is striking and supports the notion of specific structure-function relationships.

A common feature among several adhesion molecules is the presence of an Arg-Gly-Asp (RGD) sequence (26), which confers the ability to interact with specific receptors on the cell surface (*e.g.* the integrins). Such a requirement is found in neither the 18K-LAF nor in the 22-kDa mammalian homologue. Further studies are necessary to assign specific recognition sites for these two related proteins. Nevertheless, in view of the presence of the consensus sequence Glu-Asp-Arg-Arg-Trp in *Limulus* 18K-LAF which shares 95% identity with the corresponding repeating units in the mammalian protein, it is tempting to speculate that this consensus sequence assumes a key role in site-specific recognition of surface receptors thereby conferring adhesion-promoting properties to the two proteins.

There is a general concept that invertebrate species such as *L. polyphemus* cannot be viewed as prototypes of their vertebrate counterparts (27). A primitive defense mechanism is generally asssumed to exist in invertebrates, which essentially fills the void of an inducible immune system. The findings of the present paper in conjunction with studies on CRP (1, 2) and α_2-macroglobulin (25) suggest that several biologically active components from vertebrates have their counterparts in the invertebrate world. This is particularly evident when blood coagulation, complement, and adhesion processes are involved. Conversely, there is a growing body of literature that reports lectin-like activities in mammalians, which previously have been classified as primitive defense and recognition mechanisms. Highly conserved mechanisms are continuing to be discovered, and our present findings corroborate the notion that many mechanisms of self-nonself recognition co-exist in the immunological system.

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The agglutination-aggregation properties of the 18K-LAF are closely related in function to another agglutination-aggregation factor isolated from the Indian horseshoe crab, *C. rotundicauda*. However, no analysis on structural homology can be derived due to the lack of primary structure data on the *Carcinoscorpius* protein. One major difference between the two factors is that the activity of the *Carcinoscorpius* 24-kDa agglutinin is inhibited by coagulogen, while that of the 18K-LAF is not affected by coagulogen or any other proteins (including those from *Limulus* amebocyte lysate). The mechanisms underlying the agglutination and aggregation processes and their significance in defining a specific role for the horseshoe crab factor *in vivo* remains to be determined.

Whereas the 22-kDa adhesion-promoting molecule is an extracellular matrix protein, the aggregation factors from both horseshoe crabs, *L. polyphemus* and *C. rotundicauda*, were purified from amebocyte lysate as intracellular proteins. Immunocytochemical studies at the ultrastructural level, using purified rabbit anti-18K-LAF and gold anti-rabbit IgG conjugate, indicate that 18K-LAF is stored exclusively in the large dense secretory granules (Fig. 11) and is secreted to the extracellular fluid upon stimulation (*e.g.* ionophore A23187). This ionophore promotes exocytosis without cell lysis (13, 15) (data not shown). In contrast to the finding that the extracellular lectin CRP consists of a family of proteins encoded by a family of structural genes (1, 2), 18K-LAF is encoded by a single gene and a single mRNA, as indicated by Northern and Southern blot analyses (Figs. 7 and 9).

Further studies are currently in progress to elucidate the mechanism of action of 18K-LAF in *Limulus* and to define the role of the repeating units in promoting aggregation of amebocytes and/or agglutination of horse erythrocytes. Preliminary results indicate that this factor may interact with endotoxin and/or a 12-kDa endotoxin binding protein, LEBP-PI, from *Limulus* amebocyte lysate (13). The 18K-LAF protein is also retained by heparin-Sepharose (data not shown), a property shared by several adhesion molecules. The significance of these various interactions in the overall defense system of *Limulus* is the subject of ongoing investigations. The results from such studies coupled with those reported herein should provide additional insight regarding the phylogenetic development of host defense mechanisms in vertebrate and invertebrate species.

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Supplementary Material To:

Isolation, cDNA Cloning and Characterization of an 18-kDa Hemagglutinin and Amebocyte Aggregation Factor from *Limulus Polyphemus*

Nobutaka Fujii, Conceição A. S. A. Minetti, Hira L. Nakhasi, Shun-Wen Chen, Elizabeth Barbehenn, Paulo Humberto Nunes and Nga Yen Nguyen

EXPERIMENTAL PROCEDURES

MATERIALS

Live horseshoe crabs (*Limulus polyphemus*) were obtained from the Associates of Cape Cod, Inc., Woods Hole, MA. The crabs were stored in tanks and periodically fed scallops. Reagents used for amino acid sequence analysis were supplied by Applied Biosystems, Inc. Restriction enzymes T4 DNA ligase, T4 polynucleotide kinase, Klenow fragment of DNA polymerase I, *Escherichia coli* strains HB101 and JM103, M13 mp18 and mp19 replicative forms, DNA size markers, oligo (dT)-cellulose were purchased from Bethesda Research Laboratories; Guanidinium isothiocyanate was obtained from Fluka Chemical Corp. (Ronkonkoma, NY); [γ-^{32}P]ATP, specific activity 1000-3000 Ci/mmol, [α-^{32}P]dATP, specific activity 600 Ci/mmol and 1000-3000 Ci/mmol, reagents and enzymes for cDNA cloning were purchased from New England Nuclear; M13 dideoxy sequencing systems, SP-sephadex C-25 and CM-sepharose CL-6B were supplied by Pharmacia P-L Biochemicals; Nitrocellulose filters were obtained from Schleicher & Schuell; Other buffers and reagents were purchased from Burdick & Jackson, Fisher Scientific and Sigma Chemical.

METHODS

1. *Isolation and Purification of 18K-LAF:* Frozen *Limulus* amebocytes were washed in sterile 2 % saline and homogenized on ice in ten volumes of extraction buffer (0.05 M Tris-HCl, pH 7.9, 5 % Me_2SO) with a Dounce homogenizer. Cells were pelleted by centrifugation at 5,000 rpm for 30 min at 4 °C. The supernatant was fractionated sequentially by ion-exchange chromatography on SP-Sephadex C-25 and CM-Sepharose CL-6B at 4 °C, followed by gel chromatography on Sephadex G-50.

2. *Polyacrylamide Gel Electrophoresis under Denaturing Conditions:* SDS-PAGE was conducted on 14 % polyacrylamide gel (28). Vertical slab gels (15 x 18 cm) were run at a constant current of 3 mA for 16 h.

3. *Amino Acid Analysis:* Samples were hydrolyzed under vacuum in 4 N methane sulfonic acid (29). The hydrolysates were analyzed on a Beckman 6300 analyzer (30, 31). Serine, threonine and tryptophan were not corrected for destruction during hydrolysis.

4. *Automated Amino Acid Sequence Analysis:* Edman degradation of intact 18K-LAF was performed with an ABI 470A microsequencer (32). PTH-derivatives were identified on an ABI 120A analyzer (33). The concentration of starting material was determined by amino acid analysis and used to calculate the initial yields of PTH-amino acids.

5. *Digestion With Thermolysin:* Digestion of 18K-LAF with thermolysin was performed as described (1, 34) except for the following: the protein was denatured by incubation in 9 M guanidine-HCl at 50 °C for 1 hr. Enzymatic digestion (enzyme/substrate = 1:100 at 37 °C for 3 h) was carried out in a buffer containing 0.1 M Tris-HCl, pH 6.8, 5 mM Ca^{2+} and 1 M guanidine-HCl. The reaction was stopped by the addition of glacial acetic acid.

6. *Purification of Peptides:* Peptides derived from thermolysin digestion were purified by reverse phase HPLC. The fractions were subjected to performic acid oxidation followed by amino acid analysis. Cysteic acid containing fractions were further purified by HPLC. Detailed information is provided in the Legend to Fig. 10.

7. *Digestion with Carboxypeptidases:* The protein was reduced and alkylated in the presence of 50 µCi of ^{14}C-iodoacetamide (1, 34). The radiolabeled protein was digested with carboxypeptidases; samples of 1 nmol were withdrawn after 30, 60, 120, 240 min and 16 h, mixed with an equal volume of 3 % TFA, pH 1.2, and evaporated. The residue was redissolved in 0.2 M citrate buffer (pH 2.2) and subjected to amino acid analysis.

8. *Hydrazinolysis:* The intact protein was incubated with anhydrous hydrazine at 80 °C for 24 h (35). Excess hydrazine was removed and the sample dried under vacuum. The residue was redissolved in pH 2.2 buffer and subjected to amino acid analysis.

9. *Immunological Studies:* Rabbit antiserum against 18K-LAF was prepared as described for CRP (36). The antibody was purified by protein A agarose chromatography. Immunodiffusion was performed on 0.8 % agarose coated glass plates in PBS. Western blot analysis was performed as described by Minetti et. al. (13).

10. *Preparation of RNA:* *Limulus* hemolymph was collected from a pool of animals by cardiac puncture. Amebocytes were pelleted by centrifugation at 2,000 rpm for 15 min at 4 °C, and lysed in the presence of guanidinium isothiocyanate (37). Total RNA was purified by centrifugation to equilibrium in cesium chloride, followed by phenol/chloroform extraction and ethanol precipitation. Poly(A+) RNA was separated by specific adsorption to an oligo(dT)-cellulose column (38).

11. *Construction of cDNA Library:* Double-stranded cDNA was prepared as previously described (39, 40) except for the following modifications: prior to first strand synthesis, poly (A+) RNA was denatured with 10 mM methyl-mercury hydroxide at 25 °C for 10 min; the oligo(dC)-tailed double-stranded cDNA was annealed to Pst I cut, oligo(dG)-tailed pBR322 vector and recombinant plasmids were used to transform *Escherichia coli* strain HB101 (41). Ampicillin-sensitive (tetracycline-resistant) transformed colonies were selected for further hybridization to specific oligodeoxynucleotide probes (42).

12. *Synthesis of Oligodeoxyribonucleotide Probes:* Three single-stranded oligodeoxyribonucleotide probes were synthesized by the phosphoramidite method on an Applied Biosystems DNA synthesizer (43, 44). The degenerate probes correspond to the first 6 - 8 amino acids of the protein and the single-stranded probe corresponds to residues -8 to -14 of the leader peptide. DNA probes were labeled at their dephosphorylated 5'-end (45) and purified by gel filtration chromatography on a PD-10 column in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 100 mM NaCl.

13. *Screening of cDNA Library:* The cDNA library was screened as described previously (42). Hybridization of nitrocellulose filters was performed in heat-sealable bags at 42 °C, using radiolabeled probes I or II ($0.5 - 1.0 \times 10^6$ cpm/ml of hybridization solution); filters were washed at 35 °C in 15 mM sodium citrate, 0.15 M NaCl, pH 7.0 (1x SSC buffer), containing 0.1 % SDS, dried and autoradiographed at -70 °C.

14. *DNA Sequencing:* cDNA from three recombinant plasmids (pf22, pf15 and p37) were digested with Pst I and ligated to M13 mp18 and mp19 vectors (46). Competent cells (*E. coli* strain JM103) were transfected with the recombinant M13 vectors, and beta-galactosidase negative plaques were selected by hybridization with probes I or II, separately. Single-stranded cDNA prepared from M13 plaques was used as a template for dideoxynucleotide chain termination (47) in which the annealing of either mixed or single sequence probes to template was performed at 90 °C for 2 min in the presence of 1 mM EDTA.

15. *Restriction Enzyme Mapping of Recombinant DNA:* Clones which produced strong hybridization signals on duplicate filters were selected and recombinant DNA was purified by centrifugation (50,000 rpm) at 25 °C for 16 h on a cesium chloride/ethidium bromide gradient (48,49). Recombinant cDNAs were digested separately with the restriction endonuclease Alu I, EcoR I or Dde I. The cleaved DNA fragments were analyzed on an 0.8 % agarose gel, stained and transferred to nitrocellulose filters. cDNA fragments containing the sequence specific for 18K-LAF were identified by hybridization to probe I.

16. *Northern Blot Analysis:* Poly (A+) RNA was analyzed on a denaturing gel in the presence of formamide and formaldehyde (50); DNA gel markers and *E. coli* ribosomal RNAs (16S, 23S) were used as size markers. Lanes containing DNA markers were excised and stained with ethidium bromide; the remaining gel portion was subjected to Northern analysis; Northern blot was hybridized to nick-translated recombinant plasmid pf22 (8×10^7 cpm/filter). The filter was washed in 0.1 x SSC and 0.1 % SDS at 42 °C, dried and autoradiographed at -70 °C.

17. *Primer Extension:* A single-stranded seventeen base oligodeoxynucleotide primer, complementary to the mRNA and coding for amino acid residues 1-6 of the mature protein was annealed to poly (A+) RNA. Primer extension was performed as described for first strand synthesis of cDNA, in the presence of reverse transcriptase, except that primer and template were annealed prior to chain elongation. The extended primer was analyzed on a DNA sequencing gel with a cDNA of known sequence serving as size marker.

18. *Southern Blot Analysis:* Total genomic DNA was digested to completion with the restriction endonuclease Bgl II. The digest was fractionated on 0.8 % agarose gel. Southern blot (51) was hybridized to a 300 bp nick-translated probe from pf22 insert.

19. *Hemagglutination Assay:* The agglutination of a 2% suspension of horse erythrocytes by 18K-LAF was performed essentially as described (52) in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 100 mM $CaCl_2$. Hemagglutination was determined after incubation of erythrocytes in round bottom microtiter plates (1 h at 25 °C) with increasing amounts of either 18K-LAF, BSA, *Limulus* CRP or coagulogen. Inhibition of hemagglutination was determined by pre-incubating 18K-LAF with carbohydrates or other proteins prior to performing the assay.

20. *Aggregation Assay:* *Limulus* hemolymph (50 ml) was collected on ice under sterile conditions in an equal volume of pyrogen-free 3 % NaCl buffered to pH 7.2 with Tris and containing 0.1 M N-ethylmaleimide. After centrifugation (1000 rpm at 4 °C for 10 min) and fixation (30 min) in the presence of 30 mM neutralized formaldehyde (53), the cells were washed and resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl. Fifty µg of either 18K-LAF, BSA, *Limulus* CRP or coagulogen were added to 1 ml of fixed cells. Cells were observed under a phase contrast microscope at 80x-400x magnification immediately after introduction of the samples, and at time intervals of 30 min and 1 h.

21. *Electron Microscopy and Immunocytochemistry:* *Limulus* hemolymph was collected in an equal volume of 0.05 M Hepes (pH 7.4) containing 0.1 M N-ethylmaleimide, 0.165 M NaCl, 0.01 M EDTA and 0.1 % glutaraldehyde. Amebocytes were resuspended and fixed in the same buffer for 1 h. After several washes in PBS, the cells were dehydrated, embedded in "LR White" resin and sectioned (54). Following incubation, first in PBS containing 1 % BSA for 30 min and then in Rabbit anti-18K-LAF containing 20 % normal goat serum for 1 h, the grids containing the cell sections were washed in PBS and incubated with the gold-labeled second antibody conjugate. After 1 h, the cells were washed in distilled water and stained with uranylacetate (55). The sections were analyzed in a JEOL-100CX electron microscope with 8000x magnification.

22. *Exocytosis of amebocyte contents:* Amebocytes from freshly collected *Limulus* hemolymph were washed twice with sterile 0.5 M NaCl according to Armstrong & Quigley (15). Washed cells were resuspended in 0.05 M citrate buffer, 0.5 M NaCl, 10 mM EDTA and 0.1 M glucose (pH 4.6). Exocytosis was initiated by incubation with a solution containing 0.5 M NaCl, 10 mM $CaCl_2$ and 10 µM of the ionophore A23187 for 1 h at 25 °C. Expressed exocytosis fluid was separated from cell aggregates by centrifugation.

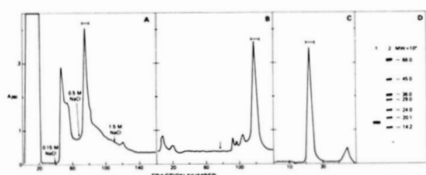


Figure 1. Purification of 18K-LAF.

Limulus amebocytes (72 g) lysed in 60 ml of buffer I (0.05 M Tris-HCl, pH 7.9, 5 % Me_2SO).

A. SP-Sephadex C-25 (2 x 10 cm), equilibrated in buffer I; Flow rate: 2 ml/min; Fraction volume: 8 ml; Temperature: 0-4 °C; Elution by stepwise gradients with 0.15 M, 0.5 M and 1.5 M NaCl in buffer I. Arrows indicate the beginning of each gradient. Fractions with hemagglutinin activity and containing 18K-LAF were pooled (bar) and subjected to CM-Sepharose CL-6B ion exchange chromatography.

B. CM-Sepharose CL-6B (2 x 55 cm), equilibrated in buffer II (0.05 M sodium acetate, pH 4.9); Sample: 18K-LAF-containing fractions from SP-Sephadex column C-25; Fraction volume: 5.0 ml; Elution by linear gradient of buffer III (1.5 M NaCl in buffer II). Other conditions and designations as in the legend to Fig. 1A.

C. Sephadex G-50 fine (1.6 x 87 cm), equilibrated in 0.1 M acetic acid; Sample: 18K-LAF- containing fractions from CM-Sepharose CL-6B; Fraction volume: 4 ml; Flow rate: 1 ml/min.

D. SDS-PAGE, Leammli buffer system; Sample: 18K-LAF, 10 µg; Current: 3 mA, 16 h; Gel concentration: 14%; MW markers: BSA (66 kD), ovalbumin (43 kD), glyceraldehyde phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), soybean trypsin inhibitor (20.1 kD) and alpha lactalbumin (14.2 kD).

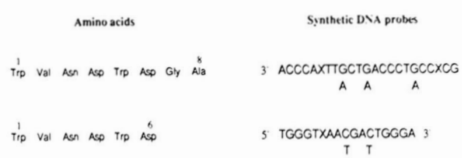


Figure 2. Nucleotide Sequence of Oligodeoxyribonucleotide Probes.

Numbers above the first and last amino acids correspond to their positions in the protein sequence; Negative numbers: leader peptide; Positive numbers: mature protein; X = ACGT.

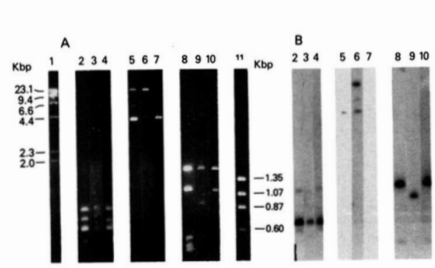


Figure 3. Restriction Enzyme Mapping of DNA from Recombinant Clones.

Recombinant DNA (0.2 µg) was digested with Alu I (lanes 2-4), EcoR I (lanes 5-7) and Dde I (lanes 8-10) and fractionated on 0.8% agarose at 30 V for 16 h.

A. Ethidium bromide stained gel. Lambda phage DNA digested with Hind III (lane 1); Recombinant DNA from clone pf22 (lanes 2, 5, 8), pf15 (lanes 3, 6, 9) and p37 (lanes 4, 7, 10); øX174 digested with Hae III (lane 11).

B. Autoradiograph of corresponding Southern blot after hybridization with ^{32}P-labeled probe I.

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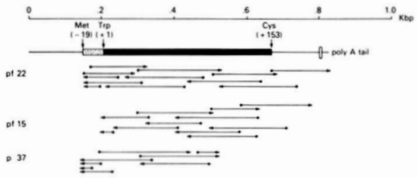


Figure 4. Sequence Strategy.

Three cDNA clones, pf22, p15 and pf37 were sequenced by the dideoxynucleotide chain termination technique. Insert DNA (0.1 µg) from the 3 recombinant plasmid clones were ligated to M13 mp18 and mp19 vectors, and transfected into E.coli strain JM 103. Single strain M13 DNA of both orientations was sequenced. A number of synthetic oligodeoxyribonucleotide primers comple- mentary to each strain were used. Arrows indicate the direction and extent of sequence resolved for each primer. SYMBOLS: Solid block: mature protein; Dotted block: leader peptide; Open box: polyadenylation addition signal; Solid line: untranslated region. Numbers in parentheses represent the positions of amino acid residues in the sequence; Negative numbers: leader peptide; Positive numbers: mature protein.

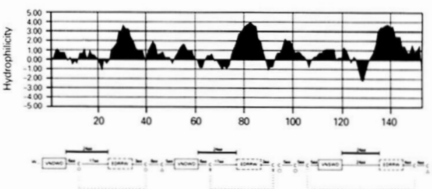


Figure 6. Hydrophilicity pattern and schematic representation of 18K-LAF three-domain structure.

Hydrophilicity scores of 18K-LAF (56) are shown in the *top frame*. Schematic representation of 18K-LAF is presented at the *bottom* where the repeating units are shown in solid and dashed boxes, respectively. Each pair of cysteine residues linked by disulfide bonds are labeled with identical symbols. The disulfide bonds which define the three looped domains are also represented as dashed lines.

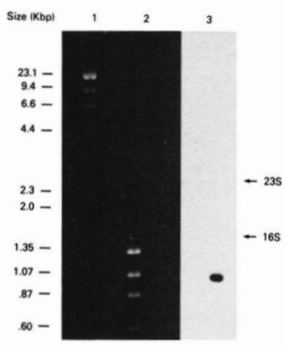


Figure 7. Northern Blot Analysis.

Sample: poly(A+) RNA, 10 µg; Running conditions: 30 V, 16 h in 1.3 % agarose, 2.2 M formaldehyde; Hybridization temperature: 42 °C; Probe: 10^8 cpm/ml of nick translated pf22 described under "Experimental Procedures"; washing: 0.1x SSC, 0.1 % SDS, 250 ml, 4 times, 42 °C. Numbers on the left: DNA size markers; Numbers and arrows on the right: 23S and 16S ribosomal RNAs; lane 1 (Lambda/ Hind III, 0.5 µg), lane 2 (Øx174 RF DNA/Hae III, 0.5 µg); lane 3: poly(A+) RNA,10 µg.

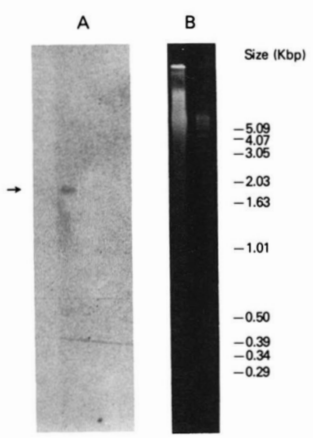


Figure 9. Southern Blot Analysis of *Limulus* Genomic DNA.

Sample: genomic DNA (20 µg) digested with Bgl II and electrophoresed on 0.8 % agarose gel, 30 V, 16 h. Hybridization temperature: 42 °C; Probe: Nick translated pf22, 10^8 cpm/filter. A. Autoradiograph of Southern blot. B. Ethidium bromide stained gel; Genomic DNA (lane 1); 1 Kb ladder size markers (lane 2, numbers on the right).

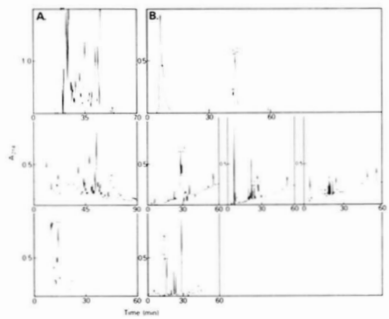


Figure 10. Purification of Disulfide Linked Peptides.

A. Upper panel: Sample: 18K-LAF (2 mg in 0.2 ml of 9 M guanidine HCl); Enzyme: thermolysin, 0.5 M Tris (pH 6.8) 20 mM $CaCl_2$; Enzyme/substrate: (1:100); Column: RO-318 (BioRad); Mobile phase: 0.1 % TFA (solvent A), 0.1 % TFA in acetonitrile (solvent B); Gradient: 15 - 50 % B, 70 min; Flow rate: 0.5 ml/min; Fraction volume: 250 µl. Center panel: Rechromatography of fractions (designated by a bar) from upper panel: 0.1 % ammonium acetate in water (solvent A); 0.1% TFA in acetonitrile (solvent B); Gradient: 0 % - 15 % B, 90 min. Lower panel: Rechromatography of fractions (designated by a bar) from middle panel: Gradient: 60 min. Other conditions as listed above. Peptide A22 yielded the amino acid sequences reported in Table IV.

B. Upper panel: Sample: 18K-LAF, 1 mg; Column: Bio-Sil TSK G2000SW; Mobile phase: 0.1% TFA in 20 % acetonitrile; Flow rate: 1 ml/min, 60 min; Fraction volume: 1.0 ml; Other conditions as listed above in Fig. 10 A. Center panel: Rechromatography of fraction 7 (left), 9 (center) and 10 (right); Mobile phase: 0.1% TFA (solvent A); 0.1 % TFA in acetonitrile (solvent B); Gradient: 0 - 80 % B, 60 min; Flow rate: 1 ml/min; Fraction volume: 1.0 ml. Peptides 9.13 (center) and 10.8 (right) yielded the amino acid sequences reported in Tables III and VI, respectively. Lower panel: Rechromatography of fraction 7.14; Gradient: 15 - 45 % B, 60 min; Flow rate; 0.5 ml/min. Fraction 7.14.5 yielded the amino acid sequences reported in Table V.

Table I: Partial Amino Acid Sequence of 18K-LAF *

Cycle No.	1				5				10					15
Residue No.	1				5				10					15
Amino acid	Trp	Val	Asn	Asp	Trp	Asp	Gly	Ala	Leu	Asn	Phe	Gln	Cys	Gln	Leu
nmol	1.8	2.1	2.8	1.7	1.4	1.8	1.2	1.7	2.7	2.3	1.9	1.4	*	2.0	2.0
	16				20					25					30
	16				20					25					30
	Lys	Asp	Ser	Ile	Lys	Thr	Ile	Ser	Ser	Ile	His	Ser	Asn	His	His
	3.0	1.3	0.3	0.8	2.2	0.4	0.6	0.2	0.2	0.4	0.2	0.1	0.6	0.6	0.7
	31				35					40					45
	31				35					40					45
	Glu	Asp	Arg	Arg	Trp	Asn	Phe	Gly	Cys	Glu	Arg	Thr	Leu	Arg	Asp
	1.6	0.5	1.1	1.4	0.3	0.5	0.3	0.2	*	0.7	0.9	0.1	0.3	0.1	0.3
	46				50					55					60
	46				50					55					60
	Pro	Ser	Cys	Tyr	Phe	Thr	Asn	Tyr	Val	Asn	Asp	Trp	Asp	Lys	Leu
	0.4	0.1	*	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.2	0.5	0.2	0.3	0.2
	61														
	61														
	Leu	His	Phe												
	0.2	0.1	0.1												

* Notes: Sample amount = 3.4 nmol; Initial yield (based on Asn[3]) = 79.4%; Repetitive yield = 96.8%; Cycles run = 74; Cycles identified = 63, ABI 470A with ABI120A

* = Identified by DNA sequence analysis and performic acid oxidation

Table II: Amino Acid Composition of 18K-LAF *

Amino acid	Number	Percent
Aspartic acid	16	10.46
Threonine	9	5.88
Serine	9	5.88
Glutamic acid	6	3.92
Glycine	7	4.58
Alanine	4	2.61
Cysteine	10	6.54
Valine	6	3.92
Isoleucine	6	3.92
Leucine	9	5.88
Proline	2	1.31
Methionine	0	0
Tyrosine	8	5.23
Phenylalanine	6	3.92
Asparagine	12	7.84
Glutamine	3	1.96
Tryptophan	8	5.23
Lysine	10	6.54
Histidine	9	5.88
Arginine	13	8.50

* Based on the cDNA deduced amino acid sequence.

Table III: Amino Acid Sequence of Disulfide-Linked (Cys^{13} - Cys^{39}) Peptide 9.13 *

Cycle No.	1				5			
Residue No.	11				15			
	37				41			
Amino acid	Phe	Gln	Cys	Gln	Leu	Lys	Asp	Ser
	Phe	Gly	Cys	Glu	Arg	Thr		
pmol	538	210	*	127	90	111	129	50
	+	292	*	157	155	132		

* Notes: Sample amount = 0.7 nmol; Initial yield (based on Phe) = 76.8%; Cycles run = 15; Cycles identified = 8

* = Analysis of performic acid oxidized peptide 9.13 yielded 1.94 nmol of cysteic acid per mole of peptide.

\+ = Combined recoveries of phenylalanine residues at positions 11 and 37.

Table IV: Amino Acid Sequence of Disulfide-Linked (Cys^{104} - Cys^{148}) Peptide A22 *

Cycle No.	1				5		
Residue No.	103				107		
	145				149		
Amino acid	Thr	Cys	Ala	Trp	Thr	Gly	Tyr
	Phe	Gln	His	Cys	Arg		
pmol	370	*	258	200	40	54	62
	550	470	116	*	90		

* Notes: Sample amount = 0.7 nmol; Initial yield (based on Phe^{145}) = 78.5%; Cycles run = 15; Cycles identified = 7

* = Analysis of performic acid oxidized peptide A22 yielded 2.2 nmol of cysteic acid per mole of peptide.

Table V: Amino Acid Sequence of Disulfide-Linked (Cys^{48} - Cys^{153}) Peptide 7.14.5 *

Cycle No.	1				5				
Residue No.	43				47				
	103				107				
	145				149				
Amino acid	Leu	Arg	Asp	Pro	Ser	Cys	Tyr	Phe	Thr
	(Thr	Cys	Ala	Trp	Thr	Gly)			
	(Phe	Gln	His	Cys	Arg)	Leu	Lys	Asn	Cys
pmol	384	132	195	178	78.8	*	156	64	54.7
	(190	*	200	167	75	77)			
	(326	202	71.2	*	111)	84.3	86.6	110	*

* Notes: Sample amount = 0.8 nmole; Initial yield (based on Leu^{43}) = 48%; Cycles run = 20; Cycles identified = 9; Amino acids and numbers within parentheses represent the disulfide-linked Cys^{104} - Cys^{148} peptide A22 reported in Table IV.

* = Analysis of performic acid oxidized peptide 7.14.5 yielded 3.6 mol of cysteic acid per mole of peptide.

Table VI: Amino Acid Sequence of Disulfide-Linked (Cys^{65} - Cys^{91}) Peptide 10.8 *

Cycle No.	1				5					10					15
Residue No.	65				69										
	77				81					86					91
Amino acid	Cys	Lys	Ser	Gly											
	Tyr	His	Asp	Asn	Arg	Arg	Glu	Asp	Arg	Arg	Trp	Lys	Ile	Tyr	Cys
nmol	*	14.9	14.2												
nmol	24.3	10.6	6.5	15.5	7.2	8.6	10.1	13.9	7.2	12.0	14.6	2.4	1.4	1.6	*

* Notes: Sample amount = 3.7 nmol; Initial yield (based on Tyr^{77}) = 65.6%; Cycles run = 23; Cycles identified = 15

* = Analysis of performic acid oxidized peptide 10.8 yielded 1.77 nmol of cysteic acid per mole of peptide.

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